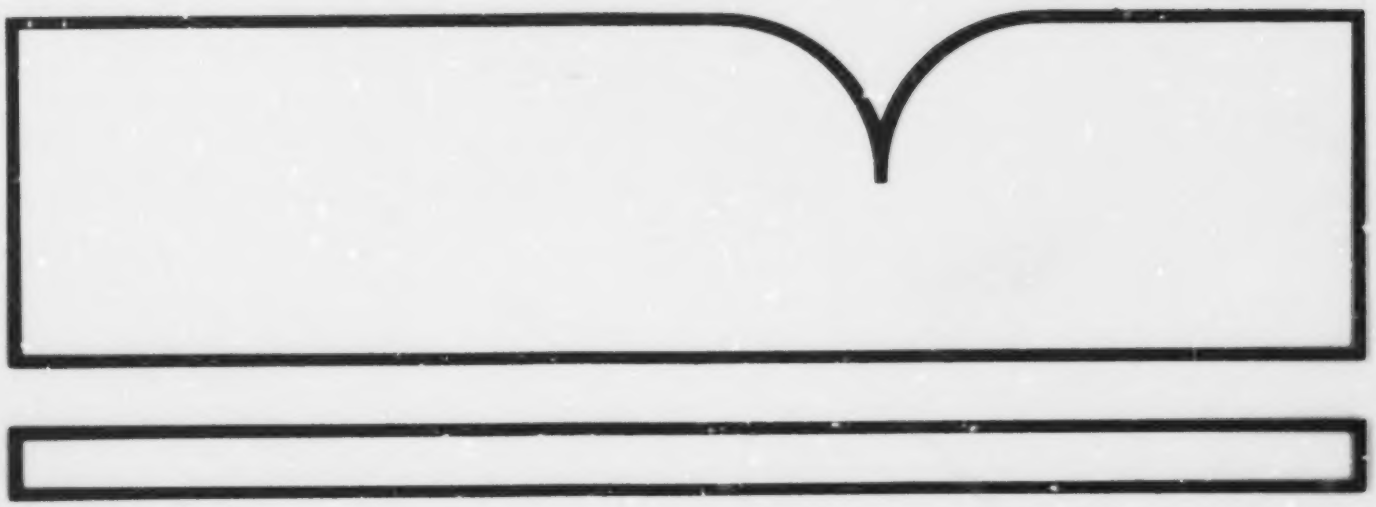
PB85-155877

Detection and Identification of 'Giardia' Cysts Using Immunofluorescence and Phase Contrast Microscopy

(U.S.) Health Effects Research Lab.
Cincinnati, OH

Jan 85



U.S. Department of Commerce
National Technical Information Service
NTIS

PB85-155877

EPA/600/D-85/020
January 1985

DETECTION AND IDENTIFICATION OF *GIARDIA* CYSTS USING IMMUNOFLUORESCENCE AND PHASE CONTRAST MICROSCOPY

by

Judith A. Sauch
Toxicology and Microbiology Division
U.S. Environmental Protection Agency
Cincinnati, Ohio 45268

HEALTH EFFECTS RESEARCH LABORATORY
OFFICE OF RESEARCH AND DEVELOPMENT
U.S. ENVIRONMENTAL PROTECTION AGENCY
RESEARCH TRIANGLE PARK, NORTH CAROLINA 27711

REPRODUCED BY
NATIONAL TECHNICAL
INFORMATION SERVICE
U.S. DEPARTMENT OF COMMERCE
SPRINGFIELD, VA. 22161

TECHNICAL REPORT DATA
(Please read instructions on the reverse before completing)

1. REPORT NO.	2.	3. RECIPIENT'S ACCESSION NO.
EPA/600/D-85/020		PB85 155877/AS

4. TITLE AND SUBTITLE	5. REPORT DATE
Detection and Identification of *Giardia* Cysts Using Immunofluorescence and Phase Contrast Microscopy	January 1985
	6. PERFORMING ORGANIZATION CODE

7. AUTHOR(S)	8. PERFORMING ORGANIZATION REPORT NO.
Judith A. Sauch	

9. PERFORMING ORGANIZATION NAME AND ADDRESS	10. PROGRAM ELEMENT NO.
USEPA, HERL, TMD, MB 26 West St. Clair Street Cincinnati, Ohio 45268	ABMC1A 11. CONTRACT/GRANT NO. In-house

12. SPONSORING AGENCY NAME AND ADDRESS	13. TYPE OF REPORT AND PERIOD COVERED
Health Effects Research Laboratory Office of Research and Development U.S. Environmental Protection Agency Research Triangle Park, NC 27711	Proceedings 14. SPONSORING AGENCY CODE EPA-600/11

15. SUPPLEMENTARY NOTES

AWWA 12th Annual Water Quality Technology Conference Proceedings, Denver, CO, December 1984.

16. ABSTRACT

Detection and identification of *Giardia* cysts in water samples has been improved by the development of an immunofluorescent method that specifically stains *Giardia* cysts bright green and allows their easy detection against a black background. The report discusses aspects of the method--reagents and instrumentation--that must be considered and optimized by anyone wanting to adopt this technique for the detection of *Giardia* cysts.

17. KEY WORDS AND DOCUMENT ANALYSIS

a. DESCRIPTORS	b. IDENTIFIERS/OPEN ENDED TERMS	c. COSATI Field/Group

18. DISTRIBUTION STATEMENT	19. SECURITY CLASS *(This Report)*	21. NO. OF PAGES
Release to Public	Unclassified	12
	20. SECURITY CLASS *(This page)*	22. PRICE
	Unclassified	

EPA Form 2220-1 (Rev. 4-77) PREVIOUS EDITION IS OBSOLETE

NOTICE

This document has been reviewed in accordance with U.S. Environmental Protection Agency policy and approved for publication. Mention of trade names or commercial products does not constitute endorsement or recommendation for use.

DETECTION AND IDENTIFICATION OF *GIARDIA* CYSTS USING IMMUNOFLUORESCENCE AND PHASE CONTRAST MICROSCOPY

Judith A. Sauch
Microbiologist

Microbiology Branch
Toxicology & Microbiology Division
Health Effects Research Laboratory
U. S. Environmental Protection Agency
Cincinnati, Ohio 45268

INTRODUCTION

The flagellated protozoan, *Giardia*, recognized now as the most common intestinal parasite in the U. S., can be transmitted via municipal water supplies and mountain streams (1). Coliform counts are unreliable as indicators for the presence of *Giardia* cysts in water supplies because the cysts are much more resistant to disinfection (2). In the absence of a cultural method, detection and identification of these protozoans has been accomplished by direct microscopic observation of sample concentrates (3). In this method, the observer must locate and identify *Giardia* cysts among numerous microorganisms of similar size and shape and against a background of debris.

Figure 1 is a phase contrast microphotograph of one field in a typical sample. An average sample would be composed of thousands of such fields. Within each field, the observer must quickly scan each object and decide if it meets the size [larger than 8 μm and smaller than 19 μm] (3) and shape requirements for a *Giardia* cyst. This is very time consuming and fatiguing. Fatigue and any distortion of a cyst from normal morphology may result in the observer not recognizing it and subsequently declaring the sample free of *Giardia*. In practice it is undesirable to use a test for regulatory purposes that can easily yield false negative results.

An immunofluorescence detection method was developed by the author and used to generate Figure 2. This is exactly the same field as seen in Figure 1. Using this method, the *Giardia* cyst in this microscopic field is now easily detectable since it glows bright green against a black background. Contaminating microorganisms and debris are either not visible or show up as dull red or light yellow objects. The observer can confirm the identity of any *Giardia* cyst-like objects found by switching objectives and using phase contrast at high magnification to locate characteristic internal morphological features (1-4 nuclei, median bodies, and axonemes).

When the amount of solids in a sample is small, it has been possible using the fluorescent antibody technique to scan the concentrate from an entire water sample of 100 or more gallons in as little as 20 minutes. The time required to scan the concentrate from a sample will vary with the quantity of particulate matter present in the concentrate. In the author's laboratory, it was determined that to examine all of the concentrate from a given water sample would take about 30 times as long using direct observation by phase as compared to the immunofluorescent method.

The focus of this paper will be to provide the reader with an introduction to the immunofluorescent method of detecting microorganisms. A paper describing the technical details for detection of Giardia cysts in water samples is in preparation.

IMMUNOFLUORESCENCE TECHNOLOGY

OVERVIEW

The Direct Fluorescent Antibody Assay. If a mammal is inoculated with proteins from a different organism, the mammal's immune system responds by producing a class of molecules known as antibodies. Each antibody molecule is capable of specifically binding to the foreign molecule (antigen) that induced its production. Thus, if a rabbit is inoculated with a highly purified preparation of Giardia cysts, in a few weeks blood serum from that rabbit will contain large amounts of antibodies which are capable of specifically binding to Giardia cysts.

Antibodies to Giardia cysts can be purified from serum and then be chemically cross-linked to a fluorescent dye. If a sample containing Giardia cysts (Figure 3A) were exposed under the proper conditions to this fluorescent-labelled specific antibody, washed free of the excess antibody and then mounted for microscopic examination, these cysts would fluoresce upon exposure to ultraviolet (UV) light. With an epifluorescent microscope (UV illumination projected through the objective lens onto the specimen) the microorganisms could then be observed to glow brightly against a black background. In the ideal situation, if the antibody used is specific enough and other test parameters have been optimized, no other microorganisms or debris in the sample would fluoresce. The assay just described is known as a direct immunofluorescent or direct fluorescent antibody assay.

The Indirect Fluorescent Antibody Assay. A modified and more versatile approach is used to label microorganisms in the indirect fluorescent antibody assay (Figure 3B). In this technique, the fluorescent dye is not cross-linked to the specific antibody (anti-Giardia cysts). Instead, a second antibody-fluorescent dye complex, the conjugate, attaches to the specific antibody. Thus, the specific, or primary, antibody acts as a bridge binding the Giardia cyst antigens and the fluorescent conjugate together. The microscopically visible end result is virtually identical for both approaches. However, the indirect method allows many specific antibody preparations to be used in a laboratory without the need to individually cross-link each one to a fluorescent dye. Only one fluorescent conjugate directed against rabbit antibody need be stocked.

The rest of this section consists of comments on the major technical considerations involved in an indirect fluorescent antibody assay. Details on fluorescent antibody technology can be found in references 4-8.

REAGENTS

Antigens and Antibodies. Only the purest possible antigen (Giardia cyst) preparations (9) should be used to stimulate antibody production. Well-purified antigen preparations can yield high titer (relative potency) specific antibody that has very minimal cross-reactivity with other microorganisms and debris in water samples.

In the author's laboratory, rabbits have been used because many animals can be easily housed and each can produce large volumes of high quality antisera. To produce the antiserum, several healthy adolescent Giardia-free rabbits housed in a well-controlled clean environment should be inoculated with antigen. Antiserum harvested from these animals should be titered, tested for major cross-reactivity and stored frozen. Detailed information on antibody production and purification can be found in references 6, 7, and 10.

The Conjugate and the Fluorochrome. Conjugates for the indirect assay consist of the fluorochrome (the label) chemically cross-linked to a species specific anti-globulin antibody (Figure 3B). The suitability of commercially-prepared conjugate from different sources for use in an indirect assay must be evaluated in each assay. In the author's experience, the ideal conjugate should be usable at a high dilution, cause little or no background fluorescence, and retain its brightness during storage and sample examination.

Fluorescein, as fluorescein isothiocyanate, has such a high efficiency of converting exciting UV light into emitted visible light that it has been almost universally adopted as the principle fluorochrome in conjugates used for immunofluorescent labeling.

The characteristic intense apple green fluorescence of fluorescein is not commonly encountered in water samples and is easily differentiated from the primarily red and light yellow autofluorescence of many microorganisms. Rhodamine is an alternate fluorochrome that fluoresces red and is widely used when another color is required for identification of a second antigen in a given sample. Sources for commercially-available fluorescein and rhodamine conjugates are listed in Linscott's Directory (11).

The Counterstain. Counterstains, such as Evans Blue, are often used in fluorescent antibody assays to improve the phase contrast or brightfield image and to minimize both non-specific binding of the conjugate and autofluorescence (6). Optimal concentrations of counterstains must be determined for use with a given specific antibody/conjugate combination, as too high a concentration results in decreased intensity of the conjugate's fluorescence.

Evaluation of Reagents. The relative concentrations of each of the reagents used in an indirect fluorescent antibody assay must be optimized. A "checkerboard" titration is performed in which systematically varied concentrations of the specific antibody, conjugate, and counterstain are used in actual assays in all reasonable combinations. The optimal combination is that which yields: (1) intense fluorescent staining of the cysts when specific antibody is used, (2) only background levels of staining when unimmunized rabbit serum is substituted for specific antibody, (3) easily visible internal morphology by phase contrast, and (4) only barely perceivable levels of nonspecific staining of other microorganisms and debris when specific antibody is used.

Mounting Media. Selection of the mounting medium for microscopic examination is very important and depends on: the substrate upon which the immunofluorescent method was performed (i.e., glass slides, membrane filters), storage time of the sample after labeling with antibody and conjugate, and the necessity to examine the sample by phase contrast microscopy. The best mounting fluid for a particular

system will not autofluoresce, will not inhibit fluorescence emission, and will allow a high contrast phase image of the microorganisms.

Exposure of the fluorescein conjugate to exciting UV light also causes rapid fading of the apple green fluorescence. However, this fading can be minimized by the incorporation of either n-propyl gallate (12) or phenylenediamine (13) into the mounting media. Optimal concentrations of these chemicals must be determined for each system as too high a concentration may completely inhibit fluorescence emission.

INSTRUMENTATION AND METHODOLOGY

The Manifold. If fluorescent antibody assays are to be run on samples supported on membrane filters, it is the author's experience that a multiport vacuum manifold be used, since this allows several samples and controls to be assayed simultaneously. The manifold should be equipped with individual vacuum shut-off valves under each filter support. In addition, it is necessary to regulate the overall degree of vacuum in the manifold so as to maintain only a minimal pressure differential across the membrane filters.

It is convenient to perform immunofluorescent assays after applying controlled amounts of sample concentrates to the surfaces of membrane filters. These are held in place on the manifold by removable weights that form wells over the membranes. The filter surfaces are then sequentially flooded with the reagents which are drawn through the membrane filter by vacuum after the appropriate incubation times. Filters should be chosen that have pore sizes small enough to retain the microorganism on the filter surface and they should be composed of a material compatible with the mounting media and observation conditions.

Positive and negative controls are included each time the manifold is used to assay water samples for Giardia cysts. Positive fluorescence and good internal morphology of G. lamblia cysts (the positive control) assures the operator that the assay has been performed correctly. The absence of fluorescing cysts on membrane filters where no cysts or samples were purposely added (negative control) indicates the lack of cross membrane contamination.

The Microscope. Since its introduction, epifluorescent (incident light, vertical) illumination has replaced transmitted light as the illumination system of choice for immunofluorescent assays. In epifluorescent illumination (Figure 4) exciting light, usually from a high pressure mercury vapor lamp (50 or 100W), passes through an exciting filter, is deflected downward by a dichroic beam splitting mirror into and through the objective to the stained specimen. Emitted and some reflected light are deflected by the dichroic mirror to the observer through a barrier filter which absorbs unwanted and harmful UV light. This results in a high level of fluorescence intensity with little bleaching of the fluorochrome in areas not yet examined. Most major manufacturers now produce microscopes with epifluorescent illumination systems and frequently it is possible to convert an existing microscope to this mode of operation.

It is also desirable to use microscope objectives and condensers that allow any Giardia cysts located by their fluorescence to also be observed by phase contrast. This will permit detection of any charac-

teristic internal morphological features that are classically used to confirm a Giardia cyst's identity.

CONCLUDING STATEMENTS

It should be appreciated that to initially develop such an assay involves many complexities only implicit in the foregoing explanation. This was especially true when the author developed an indirect fluorescent antibody assay for Giardia lamblia cysts, as the cysts cannot be generated in vitro. It was necessary, therefore, that all work be done using purified primary isolates from human feces.

Fortunately, once developed, fluorescent antibody assays are relatively easy to perform. This is reflected in their widespread use for clinical applications, usually with commercially available reagents. The author believes that should the necessary reagents become commercially available, immunofluorescent detection of Giardia lamblia cysts in water samples could become routine.

REFERENCES

1. Craun, G. F., "Waterborne Giardiasis in the United States: A Review", Am. J. Public Health, 69:817-819, 1979.

2. Hoff, J. C., Rice, E. W. and Schaefer, F. W., "Disinfection and the Control of Waterborne Giardiasis", In Proceedings of the 1984 Specialty Conference on Environmental Engineering, Pirbazari, M. and Devinny, J. S. (Eds.), pp. 239-244, ASCE, New York, 1984.

3. Jakubowski, W., "Detection of Giardia Cysts in Drinking Water. State-of-the-Art", In Giardia and Giardiasis, Erlandsen, S. L. and Meyer, E. (Eds.), Plenum, New York, 1984.

4. Cuello, A. C. (Ed.), "Immunohistochemistry", John Wiley and Sons, New York, pp. 47-82, 1983.

5. Fetaenu, A., "Labelled Antibodies in Biology and Medicine", McGraw-Hill International, New York, 2nd Edition, 1978.

6. Goldman, M., "Fluorescent Antibody Methods", Academic Press, New York, 1968.

7. Weir, D. M. (Ed.), "Immunochemistry", Volume 1 of Handbook of Experimental Immunology, Blackwell, London, 3rd Edition, Chap. 15, 1978.

8. Wick, G., Traill, K. and Schauenstein, K. (Eds.), "Immunofluorescence Technology", Elsevier, New York, 1982.

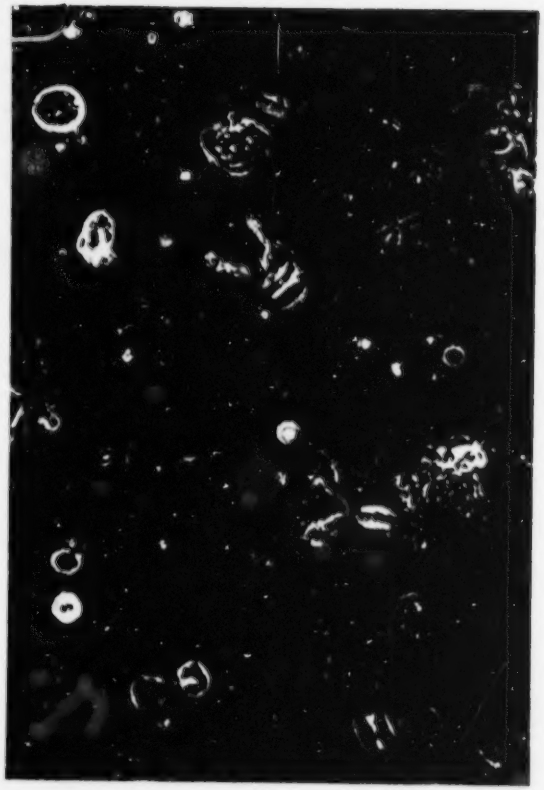
9. Sauch, J., "Purification of Giardia muris Cysts by Velocity Sedimentation", Appl. and Environ. Microbiol., 48:454-455, 1984.

10. Marchahalonis, J. J. and Warr, G. W. (Eds.), "Antibody as a Tool", John Wiley and Sons, New York, 1982.

11. Linscott's Directory of Immunological and Biological Reagents, 3rd Edition, 1984-1985.

12. Giloh, H. and Sedit, J. W., "Fluorescence Microscopy: Reduced Photobleaching of Rhodamine and Fluorescein Protein Conjugate by N-Propylgallate", Science, 217:1252-1255, 1982.

13. Johnson, L. D. and deToqueira Araujo, G. M., "A Simple Method of Reducing the Fading of Immunofluorescence During Microscopy", J. Immunol., 43:349-350, 1981.



FICURE 1. PHASE CONTRAST IMAGE OF A WATER SAMPLE (200X MAGNIFICATION)

FIGURE 2. FIELD IN FIG.1 ILLUMINATED BY ULTRAVIOLET LIGHT SHOWING A GIARDIA CYST-LIKE OBJECT NEAR CENTER

FIGURE 3. DIAGRAMATIC REPRESENTATION OF IMMUNOFLUORESCENT METHODS

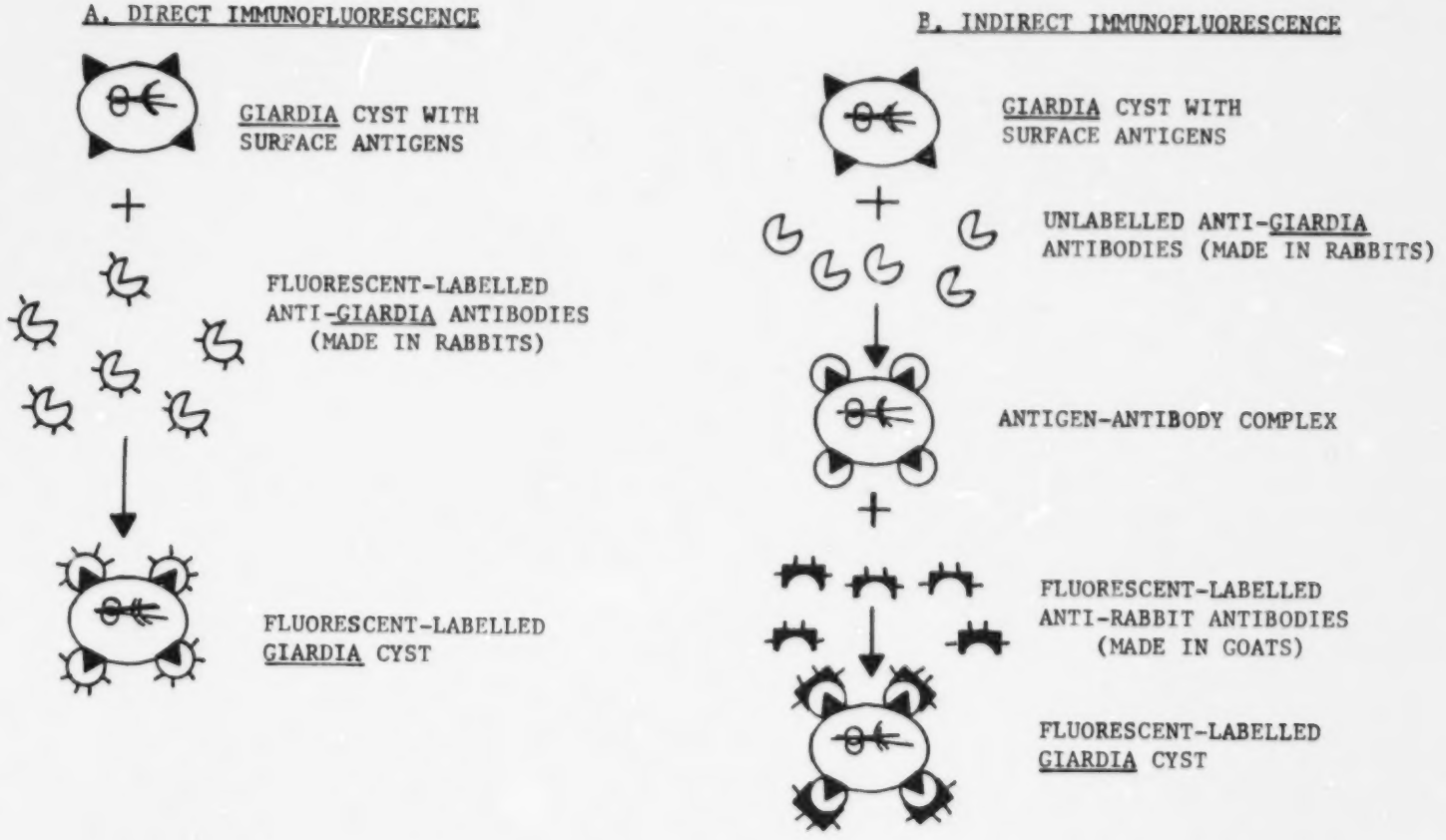

A. DIRECT IMMUNOFLUORESCENCE
GIARDIA CYST WITH SURFACE ANTIGENS
FLUORESCENT-LABELLED ANTI-GIARDIA ANTIBODIES (MADE IN RABBITS)
FLUORESCENT-LABELLED GIARDIA CYST
B. INDIRECT IMMUNOFLUORESCENCE
GIARDIA CYST WITH SURFACE ANTIGENS
UNLABELLED ANTI-GIARDIA ANTIBODIES (MADE IN RABBITS)
ANTIGEN-ANTIBODY COMPLEX
FLUORESCENT-LABELLED ANTI-RABBIT ANTIBODIES (MADE IN GOATS)
FLUORESCENT-LABELLED GIARDIA CYST

FIGURE 4. EPIFLUORESCENT ILLUMINATION